SUMMARY

1. Solutions of hydrazine catalyse the partial desulphation of the disulphate esters of glucose and galactose. Quantitative results show that, under standard conditions (pH 5·3 in 0·5 M-sodium acetate–acetic acid buffer at 38°), galactose disulphate is desulphated more rapidly than the corresponding glucose derivative and suggest that in each case one ester sulphate group only is readily removed.

2. The 6-*O*-monosulphate esters of glucose and galactose and the 3-*O*-monosulphate of glucose are not readily desulphated by hydrazine.

3. Unsuccessful attempts have been made to detect the presence in mammalian tissues of enzymes capable of liberating sulphate from monosaccharide sulphate esters, chondroitin sulphates A and C and from various sulphated oligosaccharides derived from chondroitin sulphate A.

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Glycosulphatase: Observations on the Activity of Partially Purified Preparations towards the Sulphate Esters of Certain Monosaccharides and Steroids

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The existence of glycosulphatase in the digestive organs of certain tropical marine molluscs was first noted by Soda & Hattori (1931). Between 1931 and 1948, in a series of publications (see Dodgson & Spencer, 1957*a*), Soda and his coworkers described various properties of the enzyme, although much of the quantitative work must be regarded with caution because of the impure nature of the enzyme substrate (potassium glucose monosulphate, see Dodgson & Lloyd, 1961). More recently Dodgson & Spencer (1954) showed the enzyme to be present in extracts of the digestive organs of some marine molluscs from British waters, including the common limpet (*Patella vulgata*) and the large periwinkle (*Littorina littorea*). Subsequently Roy (1956), during a study of the 3β-steroid sulphatase of *Patella*, noted that sodium cortisone 21-sulphate was desulphated by his enzyme preparations (see also Savard, Bagnoli & Dorfman, 1954) and suggested that glycosulphatase was the enzyme responsible.

In the present paper the properties of partially purified preparations of *Littorina* glycosulphatase are described. Evidence is also presented which

suggests that the ability of these preparations to hydrolyse sodium cortisone 21-sulphate cannot be attributed to the presence of glycosulphatase.

MATERIALS AND METHODS

Enzyme substrates. References to the methods of preparation of various carbohydrate and other sulphate esters used as enzyme substrates during the course of the work have previously been given (Dodgson, 1961; Dodgson & Lloyd, 1961). Sodium dehydroisoandrosterone sulphate and sodium cortisone 21-sulphate were kindly supplied by Dr A. B. Roy and Merck and Co. Inc., N.J., U.S.A., respectively. Phenolphthalein glucuronide was purchased from L. Light and Co. Ltd. and *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide was a gift from Dr P. G. Walker.

Molluscs. Supplies of *Littorina* were purchased from the local fish-market or were collected at low tide from the beach at Sully, Glamorgan. Snails (*Helix pomatia*) were obtained from L. Haig, Beambrook, Newdigate, Surrey, and were starved for 4 days before use.

Determination of the activity of various enzymes

An incubation temperature of 38° was used in all enzyme experiments.

Glycosulphatase. Glycosulphatase activity towards various monosaccharide monosulphate esters was followed by estimating enzymically liberated SO_4^{2-} ion. With crude enzyme extracts the SO_4^{2-} ion was measured by the benzidine ultramicro method of Spencer (1960) or the chloranilate procedure of Lloyd (1959). With the final *Littorina* enzyme preparation and with *Helix* preparations, the turbidimetric $BaSO_4$ procedure of Dodgson (1961) was used as described below. The buffered substrate (0·05 ml.) was incubated with an equal volume of the enzyme solution. After 1 hr., 1·4 ml. of 4% trichloroacetic acid was added and thereafter method B (see Dodgson, 1961) was followed. The concentration of enzyme was adjusted so that no more than 35 μg. of SO_4^{2-} ion was liberated and suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only just before the addition of trichloroacetic acid. Test solutions were measured against controls.

Sulphatase activity towards dehydroisoandrosterone sulphate and cortisone 21-sulphate. Lloyd's (1959) chloranilate procedure or Spencer's (1960) benzidine ultramicro method was used with crude enzyme preparations. With the purified *Littorina* preparation or with the *Helix* enzyme, the turbidimetric $BaSO_4$ method was used as described in the previous section except that the 4% trichloroacetic acid contained 12 μg. of SO_4^{2-} ion/ml. (as K_2SO_4; see Dodgson, 1961). When it was necessary to use cortisone 21-sulphate at concentrations between 0·01 and 0·02 M, 0·025 ml. of substrate was incubated with an equal volume of enzyme and, after the addition of trichloroacetic acid, 0·05 ml. of water was added to make up the appropriate volume (see Dodgson, 1961).

Arylsulphatase. A micro-adaptation of the 4-nitrocatechol method of Dodgson & Spencer (1957*b*) was used. Dipotassium 2-hydroxy-5-nitrophenyl sulphate (0·02 ml. of a 0·01 M-solution in 0·5 M-sodium acetate–acetic acid buffer, pH 5·5) was incubated with an equal volume of enzyme for 1 hr. in a 50 mm. × 60 mm. test tube. Enzyme action was stopped and the liberated 4-nitrocatechol was converted into the red anionic form by addition of 0·2 ml. of a 0·4 M-solution of glycine which had been adjusted to pH 10 by adding solid NaOH. The extinction of the solution was measured at 515 mμ (ϵ_{515} 12 400) with the Hilger model H 700.308 spectrophotometer and the 1 cm. microcells and adaptor. Suitable control determinations were made in which enzyme and substrate were incubated separately and mixed only just before the addition of the glycine buffer. Enzyme concentration was adjusted so that spectrophotometric readings ($\log I_0/I$) did not exceed 0·7.

β-Glucuronidase. The method used was a micro-adaptation of that described by Levvy (1952). Phenolphthalein glucuronide (0·02 ml. of a 2·5 mM-solution in 0·2 M-sodium acetate–acetic acid buffer, pH 4·0) was incubated for 1 hr. with an equal volume of enzyme solution. Thereafter the method followed that described in the previous section except that spectrophotometric readings were made at 554 mμ (ϵ_{554} 25 400). Suitable control determinations were made.

β-N-Acetylglucosaminidase. A micro-adaptation of the method of Findlay, Levvy & Marsh (1958) was used. *p*-Nitrophenyl *N*-acetyl-β-D-glucosaminide (0·01 ml. of a 0·01 M-solution in water) was incubated for 1 hr. with 0·02 ml. of enzyme solution and 0·01 ml. of 0·2 M-citric acid which had been adjusted to pH 4·4 by addition of 0·2 M-NaOH. Thereafter the method followed that described for arylsulphatase except that liberated *p*-nitrophenol was measured at 400 mμ (ϵ_{400} 18 200). The usual control determinations were made.

Chondrosulphatase. Ox-cartilage chondroitin sulphate A (final concn. 0·1% in 0·5 M-sodium acetate–acetic acid buffer, pH 5·0) was used as substrate and enzyme activity was followed by the method of Spencer (1960).

Chondroitinase. The method of Dodgson, Lloyd & Spencer (1957) was reduced in scale (to one-third). The assay substrate was that described in the preceding section.

Determination of nucleic acid. The ratio of the spectrophotometric readings at 280 and 260 mμ was used (Warburg & Christian, 1941).

EXPERIMENTAL AND RESULTS

Purification of Littorina *glycosulphatase*

Stage 1. The visceral organs of *Littorina* were dissected out and washed briefly in water before macerating (Townson and Mercer Ltd. macerator) for 2 min. with 5 vol. of acetone at 0°. After filtering at the pump the debris was re-macerated in fresh acetone as before, filtered and then exhaustively washed with cold acetone until the washings were colourless. The debris was dried *in vacuo* at 4° and subsequently stored at −10° until required.

Stage 2. The acetone-dried powder (10 g.) was suspended in 120 ml. of ice-cold water with the aid of a glass homogenizer. The pH of the suspension (usually approx. 9·0) was lowered to 7·0 with a few drops of acetic acid and the suspension was incubated at 38° for 15 min. Preliminary experiments showed that extraction of the acetone-dried powder with sodium acetate–acetic acid buffer at pH 7·0 was less efficient than extraction with water. After cooling to 2° the suspension was centrifuged at 6000 *g* (av.) and the resultant cloudy supernatant was held at 2° whilst the debris was washed by re-suspending it in 80 ml. of ice-cold

water and centrifuging as before. The supernatants were pooled and clarified by centrifuging at 78 000 *g* (av.) in the Spinco preparative ultracentrifuge. The clear extract was then dialysed in Visking dialysis bags (Hudes Merchandising Corp., 52 Gloucester Place, London, W. 1) for 24 hr. at 4° against several changes of water. The enzyme solution was transferred to new dialysis bags after 12 hr., otherwise a cellulase which was also present in the extract (cf. Roy, 1956) tended to rupture the dialysis tubing.

Stage 3. The dialysed extract was kept at 4° whilst the pH was lowered to 2·3 by the addition of 2N-HCl. After the extract had been standing for 2 min. the pH was quickly elevated to 4·6 with 2N-NaOH and, after standing for 2–3 hr., the whole was centrifuged at 0° and 6000 *g* (av.). This acid treatment (originally described by Dodgson & Spencer, 1953) destroyed most of the β-glucuronidase which is present in the extract and removed much other unwanted material. The cellulase was also apparently destroyed, since the extract was no longer capable of rupturing dialysis tubing. The clear supernatant was dialysed at 4° for 24 hr. against several changes of water.

Stage 4. The pH of the extract was adjusted to 6·7 and 0·4 ml. of a 5% solution of ribonucleic acid, which had been adjusted to the same pH, was added. A 2% solution of salmon-roe protamine sulphate at pH 6·7 was added until no further precipitation occurred. The precipitate was removed by centrifuging and a further 3 ml. of the protamine sulphate solution was added to the supernatant, which was then dialysed for 3 days at 4° against several changes of water. Precipitated material was removed by centrifuging and the pH of the clear supernatant elevated to 8·0. After standing for 2 hr. at 0° the excess of protamine sulphate which precipitated was removed by centrifuging and the clear supernatant was dialysed overnight at 4° against water. This stage resulted in an appreciable decrease in the nucleic acid content of the preparation.

Stage 5. Sufficient 2M-sodium acetate–acetic acid buffer, pH 5·7, was added to the extract to give a final concentration of 0·1M-acetate and the whole was cooled to 0°. Sufficient solid $(NH_4)_2SO_4$ was added (over a period of about 30 min., with gentle stirring) to give a final concentration equivalent to 55% saturation (calculated from the nomogram of Dixon, 1953, without correcting for temperature). After standing at 0° for 90 min. the precipitate was separated by centrifuging at 0° and discarded. The pH of the supernatant was lowered to 4·9 with acetic acid and sufficient $(NH_4)_2SO_4$ added at 0° to give a final concentration equivalent to 85% saturation (calculated as before). After standing for 2 hr. at 0° the precipitate was separated by centrifuging at 0° and 78 000 *g* (av.), dissolved in 10 ml. of water and dialysed for 24 hr. at 4° against several changes of water. The final enzyme solution was stored in plastic containers at −10°.

Table 1 shows the purification achieved for a preparation beginning with acetone-dried material prepared in the month of April and containing relatively low amounts of glycosulphatase. During the course of several preparations made in a period of about 18 months it has been noticed that glycosulphatase activity in *Littorina* varies considerably with season. Relatively small amounts of the enzyme can be detected in the winter months, whereas organisms collected in July have provided the most active preparation to date (approx. three times as active as the preparation described in Table 1). The purification procedure

Table 1. *Activities of some hydrolytic enzymes at various stages of the purification of extracts of the digestive organs of* Littorina littorea.

The following arbitrary experimental conditions were used to measure enzyme activities during purification: glycosulphatase, 0·02M-glucose 6-*O*-sulphate in 0·5M-tris–acetic acid buffer, pH 5·5; cortisone 21-sulphatase, 5 mM-cortisone 21-sulphate in 0·25M-sodium acetate–acetic acid buffer, pH 5·6; dehydroisoandrosterone sulphate, 2 mM in 0·25M-sodium acetate–acetic acid buffer, pH 4·5. Conditions used for assay of the other enzymes are described in the text. Enzyme activities refer to an incubation period of 1 hr. and 1 unit is equal to 1 μg. of the particular product of hydrolysis which is eventually measured (see text). With chondroitinase, liberated reducing material has been arbitrarily expressed as glucose (see Dodgson, Lloyd & Spencer, 1957).

Purification stage	Yield (g. dry wt.)	Nucleic acid (%)	Glycosulphatase (units/g.)	Cortisone 21-sulphatase (units/g.)	3-β-Steroid sulphatase (units/g.)	β-*N*-Acetylglucosaminidase (units/g.)	β-Glucuronidase (units/g.)	Arylsulphatase (units/g.)	Chondroitinase (μg. of glucose liberated/g.)	Chondrosulphatase (units/g.)
Acetone-dried powder	10	—	3 200*	—	—	—	—	—	—	—
Stage 2	3·12	5	9 700	4 600	3 190	29 300	169 000	207 000	615	0
Stage 3	1·50	4	16 650	6 260	6 500	58 000	1 950	182 000	0	0
Stage 5	0·13	<1	70 100	50 300	26 700	5 240	2 615	712 000	0	0
			85 000†							

* Measured on a dialysed suspension of the powder.

† Measured under optimum conditions.

results in the elimination of the chondroitinase and most of the β-glucuronidase and β-*N*-acetylglucosaminidase but other sulphatase enzymes are still present in appreciable amounts. Nucleic acid concentration has been considerably decreased and the preparation (which retains activity indefinitely when stored in the frozen state) is a suitable starting point for further purification studies.

Properties of the purified enzyme

Effect of substrate concentration and pH. Fig. 1 shows the effect of increasing concentrations of the 3-*O*-sulphate and 6-*O*-sulphate of glucose and the 6-*O*-sulphate of galactose on enzyme activity in the presence of 0·5M-2-amino-2-hydroxymethylpropane-1:3-diol (tris)–acetic acid buffer at the appropriate optimum pH. The curve for glucose 6-*O*-sulphate no longer exhibits the anomalies observed by Dodgson & Spencer (1954) and Dodgson & Lloyd (1961) for sodium acetate–acetic acid-buffered solutions of the enzyme. The broken line in Fig. 1 shows the corresponding curve for the same concentration of enzyme acting in the presence of 0·5M-sodium acetate–acetic acid, pH 5·8 (the optimum pH under these conditions). Tris–acetic acid buffer strongly enhances enzyme activity at the lower substrate concentrations but only slightly at higher concentrations.

The optimum substrate concentration for glycosulphatase acting on glucose 3-*O*-sulphate is in the region of 0·07M, whereas, at the same concentration of galactose 6-*O*-sulphate, maximum enzyme activity has still not been achieved. Table 2 lists the Michaelis constants (calculated from the plot of $[S]/v$ against $[S]$; Lineweaver & Burk, 1934), the optimum pH and the relative activity of the enzyme towards the three substrates. The enzyme shows greatest affinity and activity towards glucose 6-*O*-sulphate. Fig. 2 shows the effect of pH on enzyme activity towards the three substrates in the presence of tris–acetic acid buffer.

Effect of time of incubation and enzyme concentration. The results of these experiments are shown in

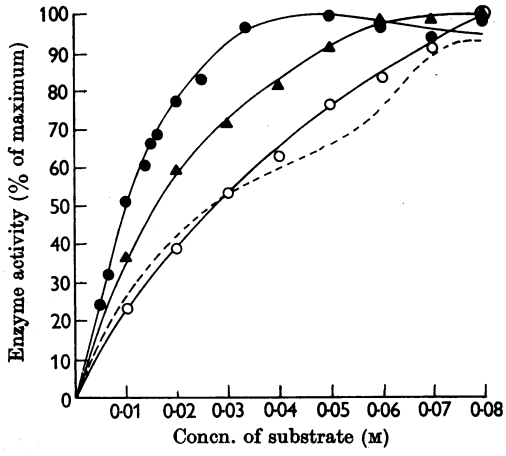

Fig. 1. Substrate concentration–activity curves for *Littorina* glycosulphatase acting on potassium glucose 6-*O*-sulphate, potassium glucose 3-*O*-sulphate and potassium galactose 6-*O*-sulphate in the presence of 0·5M-tris–acetic acid buffer at the appropriate optimum pH. ●, Potassium glucose 6-*O*-sulphate, pH 5·5; ▲, potassium glucose 3-*O*-sulphate, pH 5·8; ○, potassium galactose 6-*O*-sulphate, pH 5·2. The broken line shows the corresponding curve for the same concentration of enzyme acting on potassium glucose 6-*O*-sulphate in the presence of 0·5M-sodium acetate–acetic acid, pH 5·8.

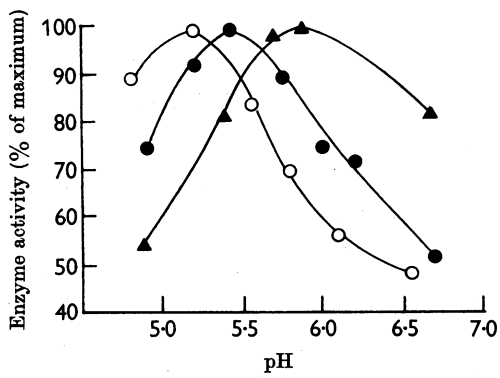

Fig. 2. Effect of pH on the activity of *Littorina* glycosulphatase towards potassium glucose 6-*O*-sulphate, potassium glucose 3-*O*-sulphate and potassium galactose 6-*O*-sulphate in the presence of 0·5M-tris–acetic acid buffer. ●, 0·04M-Potassium glucose 6-*O*-sulphate; ▲, 0·04M-potassium glucose 3-*O*-sulphate; ○, 0·04M-potassium galactose 6-*O*-sulphate.

Table 2. *Michaelis constants* (K_m), pH *optima, substrate-concentration optima and relative activities under optimum conditions for the glycosulphatase of* Littorina littorea *acting on the* 3-O- *and* 6-O-*sulphates of glucose and the* 6-O-*sulphate of galactose in the presence of* 0·5M-*tris–acetic acid buffer*

Substrate	Optimum pH	$10^2 \times$ Optimum substrate concn. (M)	$10^2 K_m$	Relative activity
Potassium glucose 3-*O*-sulphate	5·7–5·9	7·0	3·0	1·0
Potassium glucose 6-*O*-sulphate	5·5–5·6	4·0	1·7	10·0
Potassium galactose 6-*O*-sulphate	5·2	> 8·0	7·2	2·1*

* Measured at a substrate concentration of 80 mM.

Fig. 3. Glucose 6-*O*-sulphate was used as substrate at a final concentration of 0·04 M in 0·5 M-tris–acetic acid buffer, pH 5·5. Enzyme activity did not decline with time over a period of 70 min. and the enzyme concentration–activity curve provided no evidence for the presence of endogenous activators or inhibitors.

Effect of buffer concentration on glycosulphatase activity. Variation of the concentration of sodium acetate–acetic acid buffer, pH 5·8, between the limits 0·25 and 1·0 M had little effect on enzyme activity towards 0·04 M-glucose 6-*O*-sulphate. On the other hand, with tris–acetic acid buffer, pH 5·5, and the same concentration of substrate, there was a linear increase in enzyme activity with buffer concentration (overall increase of 15% between 0·25 and 1·0 M).

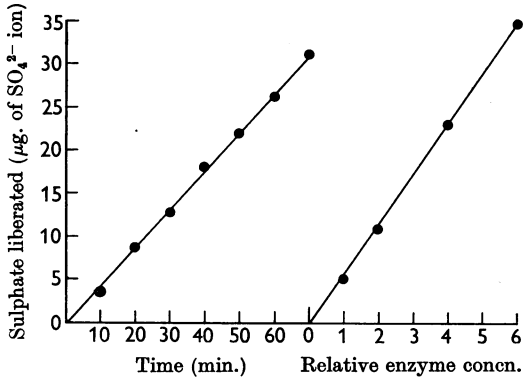

Fig. 3. Effects of time of incubation and relative enzyme concentration on the activity of *Littorina* glycosulphatase towards 0·04 M-potassium glucose 6-*O*-sulphate in the presence of 0·5 M-tris–acetic acid, pH 5·5.

Table 3. *Effects of various compounds on the activity of the glycosulphatase of* Littorina littorea

Results are expressed as a percentage of the enzyme activity obtained towards potassium glucose 6-*O*-sulphate (0·04 M in 0·5 M-tris–acetic acid buffer, pH 5·5) in the absence of added activator or inhibitor. Except where otherwise stated, the final concentration of inhibitor or activator was 0·02 M. All inhibitor and activator solutions were adjusted to pH 5·5 with a trace of M-acetic acid or -NaOH before use.

Compound added	Enzyme activity (%)
KCN	110
NaF	27
NaCl	102
$MgCl_2$	112
$MnCl_2$	120
$ZnCl_2$	88
NaH_2PO_4–Na_2HPO_4	3
$Na_4P_2O_7$*	1
Hydroxylamine	91
Semicarbazide	71
Sodium ethylenediaminetetra-acetate	104

* Final concentration 0·01 M.

Effect of various compounds on enzyme activity. Table 3 shows the effects of some common enzyme inhibitors and activators on enzyme activity towards 0·04 M-glucose 6-*O*-sulphate in the presence of 0·5 M-tris–acetic acid buffer, pH 5·5. In common with most other sulphatase enzymes, glycosulphatase was strongly inhibited by phosphate and pyrophosphate and to a less extent by fluoride. Some increase in enzyme activity was observed in the presence of $MgCl_2$ and $MnCl_2$.

Activity of the enzyme preparation towards sodium cortisone 21-sulphate and sodium dehydroiso-androsterone sulphate

The final enzyme preparation still retained activity towards cortisone 21-sulphate and dehydro-*iso*androsterone sulphate, and Figs. 4 and 5 show the effect of substrate concentration and pH on enzyme activity towards these substrates in the presence of 0·5 M-tris–acetic acid and sodium acetate–acetic acid buffers respectively. With dehydro*iso*androsterone sulphate the method of assay did not allow the use of substrate concentrations below 1·5 mM (see Dodgson, 1961). Roy (1956) has noted that the optimum concentration of this substrate for the 3β-steroid sulphatase of *Patella vulgata* is about 0·2 mM. The curve shown in Fig. 4 therefore presumably reflects the inhibition of enzyme activity by excess of substrate.

The probability that different enzymes are responsible for the hydrolysis of dehydro*iso*androsterone sulphate and cortisone 21-sulphate by *Patella* preparations has already been indicated by Roy (1956). This author suggested that glycosulphatase might be the enzyme responsible for the

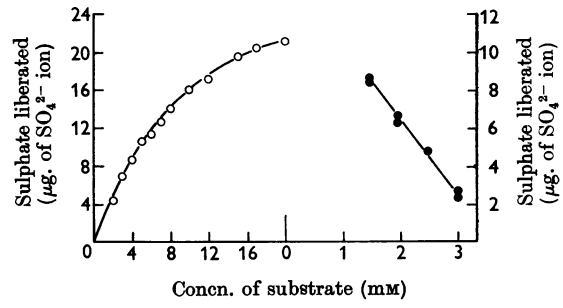

Fig. 4. Substrate concentration–activity curves for *Littorina* preparations (stage 5) acting on sodium cortisone 21-sulphate and sodium dehydro*iso*androsterone sulphate. ○, Cortisone 21-sulphate in the presence of 0·5 M-tris–acetic acid buffer, pH 5·1; ●, dehydro*iso*androsterone sulphate in the presence of 0·5 M-sodium acetate–acetic acid buffer, pH 4·5.

hydrolysis of the cortisone ester. Table 4 shows the relative rates of hydrolysis of glucose 6-*O*-sulphate, cortisone 21-sulphate and dehydro*iso*androsterone sulphate by four separate *Littorina* preparations (stage 5) under standard experimental conditions. The variation in relative activities from preparation to preparation suggests that the three substrates are hydrolysed by different enzymes.

Mixed-substrate experiments. Attempts were made to confirm the existence of three distinct enzymes by mixed-substrate experiments. The theoretical background to this type of experiment has been outlined by Dixon & Webb (1958) and by Laidler (1958). If one enzyme is responsible for the hydrolysis of two different substrates then each will act as a competitive inhibitor of the other when both are present in the same incubation mixture. Overall enzyme activity will therefore be less than the sum of the activities when each substrate is incubated separately with enzyme. In contrast, if two enzymes are involved, the overall activity when two substrates are present should approximate to the sum of the activities obtained when the two substrates are incubated separately with the same amount of enzyme.

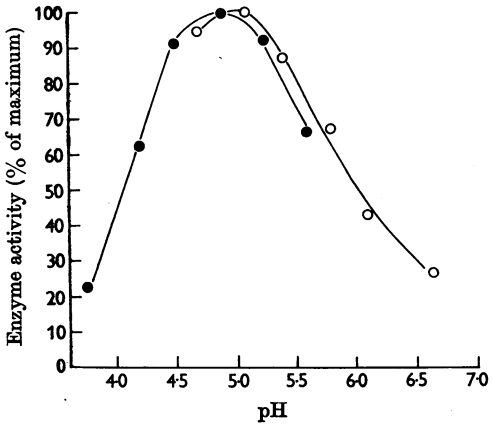

Fig. 5. Effect of pH on the activity of *Littorina* preparations towards sodium cortisone 21-sulphate and sodium dehydro*iso*androsterone sulphate. ○, 0·01 M-Cortisone 21-sulphate in 0·5 M-tris–acetic acid buffer; ●, 2 mM-dehydro-*iso*androsterone sulphate in 0·5 M-sodium acetate–acetic acid buffer.

The following procedure was used in experiments with cortisone 21-sulphate and glucose 6-*O*-sulphate. Each substrate (at a concentration four times as great as that required in the final incubation mixture) was dissolved in 1 M-tris–acetic acid buffer, pH 5·6. A portion of each stock solution was removed and diluted with an equal volume of 1 M-tris–acetic acid buffer, pH 5·6, and a 'mixed-substrate' solution was prepared by taking a further portion of one of the stock solutions and diluting it with an equal volume of the other stock solution. A portion (0·025 or 0·05 ml.) of each of the three substrate solutions thus obtained was incubated with an equal volume of the enzyme preparation (usually diluted two- or three-fold with water) for 1 hr. at 38°. Liberated SO_4^{2-} ion was then estimated in the usual way, except that the trichloroacetic acid contained 12 μg. of SO_4^{2-} ion (as K_2SO_4)/ml. A similar procedure was used with other substrate combinations except that the conditions of buffer and pH were sometimes different. In all cases, preliminary experiments were made in order to check that SO_4^{2-} ion could be estimated under the experimental conditions used.

Table 5 records the results of experiments in which the combinations of substrates were glucose 6-*O*-sulphate and cortisone 21-sulphate, dehydro-*iso*androsterone sulphate and cortisone 21-sulphate, and dehydro*iso*androsterone sulphate and glucose 6-*O*-sulphate. The amount of SO_4^{2-} ion liberated from glucose 6-*O*-sulphate and cortisone 21-sulphate, when both are present in the same incubation mixture, agrees very closely with the sum of the amounts liberated when each substrate is incubated separately, and this is good evidence that the two substrates are hydrolysed by different enzymes. Similar conclusions can be drawn for glucose 6-*O*-sulphate and dehydro*iso*androsterone sulphate. On the other hand, the results with dehydro*iso*androsterone sulphate and cortisone 21-sulphate suggest that the same enzyme is attacking both substrates. However, Dixon & Webb (1958)

Table 4. *Variation in the relative activities of four different* Littorina *preparations towards glucose 6-O-sulphate, dehydroisoandrosterone sulphate and cortisone 21-sulphate*

Experimental conditions were: cortisone 21-sulphate (CS), 0·01 M in 0·5 M-tris–acetic acid, pH 5·1; dehydro-*iso*androsterone sulphate (DHAS), 2 mM in 0·25 M-sodium acetate–acetic acid, pH 4·5; glucose 6-*O*-sulphate (GS), 0·04 M in 0·5 M-tris–acetic acid, pH 5·6.

Enzyme preparation	Date of collection of organisms	Relative activity towards CS	DHAS	GS
1	13 Oct. 1959	4·3	1·0	9·9
2	26 Oct. 1959	2·4	1·0	3·8
3	5 Nov. 1959	3·5	1·0	3·8
4	4 Apr. 1960	3·3	1·0	6·3

state: 'When additive effects are obtained, it is good evidence that two independent enzymes are concerned, but less-than-additive effects are not conclusive evidence for identity, since each enzyme might be competitively inhibited by the substrate of the other'. Inhibition of this type could well explain the experimental results obtained with dehydro*iso*androsterone sulphate and cortisone 21-sulphate.

Observations with other enzyme preparations. The digestive juice of the snail, *Helix pomatia*, contains 3β-steroid sulphatase (Henry & Thevenet, 1952), and attempts were made to test the ability of this juice to hydrolyse glucose 6-*O*-sulphate and cortisone 21-sulphate. Unpublished observations from these laboratories have shown that incubation of the juice alone results in the liberation of small amounts of SO_4^{2-} ion from endogenous sulphated

Table 5. *Results of 'mixed-substrate' experiments with* Littorina *and* Helix *preparations*

See text for explanation of these experiments. The amount of SO_4^{2-} ion liberated enzymically when two substrates are present in the same incubation mixture should be compared with the sum (figures in parentheses) of the amounts liberated when the same two substrates are incubated separately. The following abbreviations are used: CS, cortisone 21-sulphate; GS, glucose 6-*O*-sulphate; DHAS, dehydro*iso*androsterone sulphate.

Enzyme	Substrate present in the incubation mixture	Concn. of substrate in incubation mixture (mM)	Buffer	pH	SO_4^{2-} ion liberated (μg.) From 'mixed substrate'	SO_4^{2-} ion liberated (μg.) From individual substrates
Littorina littorea	CS	20	0·5M-Tris–acetic acid	5·6	—	11·9
	GS	40			—	12·6
	CS and GS	—			23·6	(24·5)
	CS	20	0·5M-Tris–acetic acid	5·6	—	12·2
	GS	60			—	13·6
	CS and GS	—			23·8	(25·8)
	CS	10	0·5M-Tris–acetic acid	5·1	—	13·2
	DHAS	2			—	8·5
	CS and DHAS	—			11·6	(21·7)
	CS	10	0·5M-Sodium acetate–acetic acid	5·1	—	18·3
	DHAS	2			—	13·2
	CS and DHAS	—			11·4	(31·5)
	DHAS	2	0·5M-Tris–acetic acid	5·1	—	7·2
	GS	20			—	13·3
	DHAS and GS	—			19·7	(20·5)
Helix pomatia (digestive juice)	CS	10	0·5M-Sodium acetate–acetic acid	5·1	—	17·8
	DHAS	2			—	1·4
	CS and DHAS	—			11·9	(19·2)

Table 6. *Activity of some* Helix *preparations towards cortisone 21-sulphate, dehydro*isoandrosterone *sulphate, glucose* 6-O-*sulphate and galactose* 6-O-*sulphate*

Experimental conditions were: cortisone 21-sulphate (CS), 0·01M, in 0·5M-tris–acetic acid buffer, pH 5·1; dehydro*iso*androsterone sulphate (DHAS), 2 mM in 0·5M-sodium acetate–acetic acid buffer, pH 4·5; glucose 6-*O*-sulphate (GS), 0·04M in 0·5M-tris–acetic acid buffer, pH 5·5; galactose 6-*O*-sulphate (Gal.S), 0·04M in 0·5M-tris–acetic acid buffer, pH 5·2. The digestive-juice preparations were diluted 40-fold before use and the digestive-gland arylsulphatase preparation (Dodgson & Powell, 1959) was used without further dilution. Incubation mixtures (0·1 ml.) contained 0·05 ml. of enzyme solution and incubation was for 2 hr. at 38°.

Enzyme preparation	SO_4^{2-} ion (μg.) liberated by 0·05 ml. of enzyme in 2 hr. from CS	DHAS	GS	Gal.S
Digestive juice (July)	28·6	<1·0	0	0
		<1·0*		
Digestive juice (Sept.)	36·7	1·1	0	0
		1·5*		
Digestive-gland arylsulphatase preparation	13·4	<1·0	0	—

* Incubation mixture consisted of 0·1 ml. of enzyme and 0·1 ml. of a 1·2 mM-solution of dehydro*iso*androsterone sulphate in 0·8M-sodium acetate–acetic acid buffer, pH 4·5, and liberated SO_4^{2-} ion was measured by the chloranilate procedure of Lloyd (1959). Results are corrected to 0·05 ml. of enzyme solution.

materials. Before use, therefore, the juice was incubated for 24 hr. at 38° in the presence of sodium acetate–acetic acid buffer (final concn. approx. 0·15 M), pH 5·6, and subsequently dialysed at 4° for 24 hr. against several changes of water.

The treated juice was usually diluted (with water) 30- or 40-fold for the enzyme experiments. Enzyme activity was measured in the usual way (Dodgson, 1961), except that with one of the two preparations used, incubation with dehydro*iso*androsterone sulphate resulted in the appearance of a faint turbidity. This turbidity did not appear in the controls where enzyme and substrate were incubated separately. Further controls were necessary to compensate for this turbidity and in practice these consisted of an additional series of test and control determinations in which a 0·5 % solution of gelatin was substituted for the $BaCl_2$–gelatin reagent during the estimation of the liberated SO_4^{2-} ion. The difference in the spectrophotometric readings (usually approx. 0·025) between these additional tests and controls was deducted from that obtained when the normal test solutions were measured against the normal controls.

Table 6 records the relative activities of two separate enzyme preparations (obtained from snails collected in July and September respectively) towards glucose 6-*O*-sulphate (0·04 M, in 0·5 M-tris–acetic acid buffer, pH 5·5), galactose 6-*O*-sulphate (0·04 M, in 0·5 M-tris–acetic acid, pH 5·2), dehydro*iso*androsterone sulphate (2 mM, in 0·5 M-sodium acetate–acetic acid buffer, pH 4·5) and cortisone 21-sulphate (0·01 M, in 0·5 M-tris–acetic acid buffer, pH 5·1). Cortisone 21-sulphate was rapidly hydrolysed by both preparations but activity towards dehydro*iso*androsterone sulphate was very low. 'Mixed-substrate' (dehydro*iso*androsterone sulphate and cortisone 21-sulphate) experiments gave results (Table 5) which were similar to those obtained with the *Littorina* preparation. No enzyme activity towards the 6-*O*-sulphates of glucose and galactose could be detected in either *Helix* preparation. It was possible that endogenous factors were inhibiting glycosulphatase activity but separate experiments showed that no significant reduction in the activity of *Littorina* glycosulphatase occurred when the *Helix* preparation was also present in the incubation mixture.

Further experiments were made with a partially purified preparation of the arylsulphatase of the digestive gland of *Helix* (see Dodgson & Powell, 1959). This preparation again exhibited appreciable activity towards cortisone 21-sulphate but little or no activity towards dehydro*iso*androsterone sulphate or glucose 6-*O*-sulphate (Table 6).

Activity of Littorina *and* Helix *preparations towards other sulphate esters*

Littorina and *Helix* (digestive juice) preparations were without effect on the sulphate esters listed in Table 7. The various experimental conditions used are also recorded in this table. The results confirm the findings of other workers (e.g. Roy, 1956) that mollusc preparations do not contain a general 'alkylsulphatase'.

Table 7. *Sulphate esters which were not hydrolysed by* Littorina *or* Helix *preparations*

In most cases incubation mixtures consisted of 0·05 ml. of enzyme solution (diluted threefold for *Littorina* and 30-fold for *Helix*) and an equal volume of a 0·08 M-solution of the sulphate ester in 1 M-tris–acetic acid buffer, pH 5·5. Sulphate esters of hydroxylated amino acids were used at a concentration of 0·04 M in the same buffer at pH 5·7 and 8·5. Incubation periods varied between 16 and 24 hr. The sulphate esters were added as the potassium salts.

Methyl sulphate
Ethyl sulphate
Ethanolamine sulphate
*iso*Propyl sulphate
1-Aminopropan-2-ol sulphate
n-Butyl sulphate
sec.-Butyl sulphate
n-Pentyl sulphate
n-Hexyl sulphate
*cyclo*Pentyl sulphate
*cyclo*Hexyl sulphate
Tetrahydrofuran 2-methyl sulphate
Tetrahydropyran 2-methyl sulphate
Propane-1:3-diol disulphate
Serine *O*-sulphate
Threonine *O*-sulphate
Hydroxyproline *O*-sulphate

DISCUSSION

Previous work from these and other laboratories has shown the potential value of enzymes as tools in the elucidation of the structures of sulphated polysaccharides (see Dodgson & Lloyd, 1958; Takahashi, 1960*a*). Glycosulphatase may be of some use in this type of study, and one of the aims of the present work has been to obtain preparations of glycosulphatase which are relatively free from other enzymes which might be active towards such polymers. The purification procedure described here results in the elimination of the chondroitinase (aminopolysaccharase) and most of the β-glucuronidase and β-*N*-acetylglucosaminidase which are initially present in *Littorina* extracts and which can participate in the degradation of chondroitin sulphates A and C. The resulting preparation is quite stable when stored in the frozen state and can be regarded as suitable starting material for further purification.

Littorina glycosulphatase is particularly active towards glucose 6-*O*-sulphate but shows relatively low activity towards glucose 3-*O*-sulphate and galactose 6-*O*-sulphate. Earlier work (Soda, 1936), in which relatively crude and ill-defined preparations of various carbohydrate sulphate esters were used, indicated that the glycosulphatase of *Charonia lampas* also showed greatest activity towards a substrate preparation now known to

have consisted mainly of glucose 6-*O*-sulphate. Recently, Takahashi (1960*b*) has purified the *Charonia* enzyme 160-fold by chromatography on carboxymethylcellulose columns. The purified enzyme exhibits properties which are very similar to those of the *Littorina* enzyme.

The anomalies in the substrate concentration–activity curve obtained when the enzyme is acting on glucose 6-*O*-sulphate in the presence of sodium acetate–acetic acid buffer (Dodgson & Lloyd, 1961) do not appear with tris–acetic acid buffers. Other partially purified sulphatase enzymes exhibit similar anomalies in the presence of sodium acetate–acetic acid buffers (see Dodgson & Wynn, 1958). It is probable that these effects merely reflect the comparative impurity of the enzyme preparations since in one case (human-liver arylsulphatase B, Dodgson & Wynn, 1958) the anomalies disappeared on further purification.

The finding of sulphatase activity towards cortisone 21-sulphate in *Patella* (Roy, 1956), *Otala* (Savard, Bagnoli & Dorfman, 1954), *Littorina* and *Helix*, the only organisms examined up to the present time, may well point to a widespread distribution of such activity in molluscs. The present work shows that the enzyme responsible for the hydrolysis of this substrate cannot be identified with glycosulphatase or chondrosulphatase. The results of 'mixed-substrate' experiments with dehydro*iso*androsterone sulphate and cortisone 21-sulphate, although suggesting that one enzyme acts on both substrates, can also be interpreted as arising from competitive inhibition of each of two distinct enzymes by the substrate of the other. The variation in the relative activities towards dehydro*iso*androsterone sulphate and cortisone 21-sulphate exhibited by *Patella*, *Littorina* and *Helix* preparations (see Roy, 1956, and Tables 4 and 6) strongly suggests that two different enzymes are involved. Roy (1956) has already pointed out that the high specificity of 3β-steroid sulphatase for the 3β-sulphate esters of 5α- and Δ^5-steroids makes it unlikely that the enzyme could be responsible for the hydrolysis of cortisone 21-sulphate.

Arylsulphatase (Dodgson, Lewis & Spencer, 1953; Dodgson & Powell, 1959), cellulose polysulphatase (Takahashi & Egami, 1960) and myrosulphaminase (myrosulphatase; see Takahashi, 1960*c*) also occur in some molluscs, and the possibility that these enzymes can attack cortisone 21-sulphate cannot yet be discounted. However, with *Patella* concentrates Roy (1956) has obtained some degree of separation of arylsulphatase from the enzyme hydrolysing cortisone 21-sulphate, by means of paper electrophoresis. Cellulose polysulphatase is known to be only slightly inhibited by 0·02M-sodium fluoride (Takahashi & Egami, 1960), whereas enzyme activity towards cortisone 21-sulphate is completely inhibited under these conditions (K. S. Dodgson, unpublished observations). Myrosulphaminase, an enzyme liberating sulphuric acid from mustard-oil glycosides, is concerned with the rupture of a N–O–S linkage and from theoretical grounds would hardly be expected to inhibit activity towards substrates containing C–O–S linkages.

Leon, Bulbrook & Corner (1960) have reported that some molluscs (including *Helix*) possess sulphatase activity towards aetiocholanolone sulphate which cannot be attributed to 3β-steroid sulphatase. However, this enzyme was not present in *Littorina*. The probability exists therefore that the enzyme acting on cortisone 21-sulphate is a new type of sulphatase, but much work remains to be done before this can be established with certainty.

The digestive juice of *Helix pomatia* is extremely active towards cortisone 21-sulphate. At pH 5·1, in the presence of 0·5M-tris–acetic acid, the activity towards 0·01M-cortisone 21-sulphate of the more active of the two preparations tested corresponded to a liberation of approx. 43 000 μg. of SO_4^{2-} ion/ml. of undiluted preparation/hr.

SUMMARY

1. The glycosulphatase of the digestive organs of the common periwinkle, *Littorina littorea*, has been purified 20-fold. Chondroitinase, β-glucuronidase and β-*N*-acetylglucosaminidase, which are also present in aqueous extracts of the digestive organs, are eliminated or greatly decreased in concentration during the purification procedure.

2. The potassium salts of the 3-*O*-sulphate and 6-*O*-sulphate esters of glucose and the 6-*O*-sulphate esters of galactose have been used as substrates for glycosulphatase. The enzyme shows greatest affinity and activity towards glucose 6-*O*-sulphate.

3. The final enzyme preparation, which also contains appreciable amounts of arylsulphatase and 3β-steroid sulphatase, is able to hydrolyse sodium cortisone 21-sulphate. Mixed-substrate experiments provide no support for the suggestion (Roy, 1956) that glycosulphatase is the enzyme responsible for the hydrolysis of cortisone 21-sulphate.

4. Preparations of the digestive juice of *Helix pomatia*, when diluted 40-fold, show little or no activity towards glucose 6-*O*-sulphate and dehydro*iso*androsterone sulphate but are strongly active towards cortisone 21-sulphate.

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The Occurrence of Unusual Fatty Acids in Faecal Lipids from Human Beings with Normal and Abnormal Fat Absorption

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Little information has been published on the detailed fatty acid composition of human faecal lipids, though it has long been a vexed question whether or not the microbial flora of the intestinal tract play a major part in the production of the large amounts of fat excreted in diseases such as steatorrhoea or sprue. Studies relating the composition of faecal lipids to that of the ingested fat should provide some information on this problem.

The earlier methods of fatty acid analysis used in such studies, e.g. fractional distillation (Edwards & Cook, 1951) and reversed-phase liquid–liquid chromatography (Van de Kamer, Pikaan, Bolssens-Frankena, Couvée-Ploeg & van Ginkel, 1955), are not capable of providing sufficiently detailed information. We have used gas–liquid chromatography both for analysis (James & Martin, 1956) and for structure determination (James & Webb, 1957; James, 1959).

EXPERIMENTAL

Faeces were collected and either extracted immediately with the medium described by Dole (1956) or stored at 2° until required. The extracted lipids were divided into free and bound acids by the titration technique described by Dole (1956).

The free acids were methylated directly with anhydrous methanolic HCl (James, Lovelock, Webb & Trotter, 1957) and the bound acids were methylated after saponification and extraction. Analyses of the methyl esters of the fatty acids were carried out on 80–100 μg. samples on 4 ft. glass columns with stationary phases of Apiezon L grease (Shell Chemicals Ltd.) at 200° with a column efficiency of 4000–5000 theoretical plates, and also polyethylene glycol adipate at 180° with an efficiency of 3000–3500 theoretical plates. In both cases the argon ionization monitor was used as detector (Lovelock, 1958; Lovelock, James & Piper, 1959; and Pye Scientific Instruments Ltd.). Isolation of individual acids on the milligram scale was carried out on similar columns with the gas-density meter as